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**"Immobilization of Enzymes in Nanoporous Host Materials:
A Nanobiotechnological Approach to Decontamination and Demilitarization
of Chemical and Biological Warfare Agents"**

Submitted by

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PREFACE

This is the final technical report on a project sponsored by US Army Research Office under Research Agreement No. DAAD19-01-1-0685. The report has been resulted from a collaborative research work between Drexel University, US Army Research Laboratory and Edgewood Chemical Biological Center. The Drexel University team includes Dr. Yen Wei, Principal Investigator on this project, Dr. Hua Dong and Mr. Zhengfei Sun. The Army team includes Dr. Ray Yin, Ms. Kate Ong and Dr. Tu-Chen Cheng. The text in this report is mostly adopted from Dr. Hua Dong's Ph.D. dissertation (Chapter 6, Drexel University, March 2002). It is noted that Ms. Ong is also a doctoral candidate at Drexel University working under the supervision of both Dr. Wei and Dr. Yin, who holds a Visiting Professor appointment at Drexel University. The project has been monitored by Dr. Stephen Lee of ARO, for whose guidance, assistance and support we are most grateful.

SECTION I. Summary

As proposed, we have established the concept of a novel nanobiotechnology for decontamination and demilitarization of chemical and biological warfare agents, based on immobilizing decon/demil effective enzymes such as organophosphorus acid anhydrolases (OPAA) in nanoporous host materials such as silica. The immobilization has been achieved by encapsulating the enzymes in nanoporous silicate or organically modified silicate frameworks prepared by the nonsurfactant-templated sol-gel technology recently developed in our laboratory. Such enzyme (guest)-nanoporous silica (host) systems offer many advantages over the use of free enzymes, including: (1) significantly improved thermal stability; (2) retain enzymatic activity in decon/demil media that contain organic solvents; (3) longer shelf life and higher storage stability; and (4) reusability and recyclability of the enzymes. Based on our results using diisopropyl fluorophosphate (DFP) as the simulant for G-type agents, encapsulated OPAA not only retains its enzymatic activity to a significant extent but also exhibits much enhanced stability. Further investigation would lead to practical applications of these novel nanobiomaterials in decontamination creams/lotions and as survivable bio-decontaminants under battlefield conditions.

SECTION II. Detailed Results, Discussion and Experimental Methods

1.0. Introduction

Immobilization of enzymes and other active biological molecules in porous inorganic silica materials via the sol-gel process¹ has drawn great interest in recent years. An array of substances, including catalytic antibodies, DNA, RNA, antigens, live bacterial, fungal, plant and animal cells, and whole protozoa, have been encapsulated in silica,²⁻¹³ organosiloxane¹⁴ and hybrid sol-gel materials.^{3,6} Sol-gel immobilization leads to the formation of advanced materials that retain highly specific and efficient functionality of the guest biomolecules within the stable host sol-gel matrix. The protective action of the sol-gel cage prevents leaching and enhances their stability significantly. The advantages of these 'living ceramics' might give them applications as optical and electrochemical sensors, diagnostic devices, catalysts, and even bio-artificial organs.^{5,6} With rapid advances in sol-gel precursors, nanoengineered polymers, encapsulation protocols and fabrication methods, this technology promises to revolutionize bioimmobilization.

Biosensors using immobilized receptors are finding ever-increasing application in a wide variety of fields such as clinical diagnostics, environmental monitoring, food and drinking water safety, and illicit drug monitoring.^{6,15-28} One of the most challenging aspects in development of these sensors is immobilization and integration of biological molecules in the sensor platform. Numerous techniques, including physical adsorption, covalent attachment, entrapment in polymer and inorganic matrices, have been explored over the past decade. Porous silicate materials made by low temperature sol-gel process are promising host matrices for encapsulation of biomolecules such as enzymes, antibodies, and cells. These sol-gel matrices are chemically inert, hydrophilic, biocompatible and inexpensive to produce. They also exhibit superior mechanical strength, enhanced thermal stability, and negligible swelling in solvents compared to organic polymers. The sol-gel matrix can also be tailored by introducing the different functional groups thereby improve the microenvironment and maintain the biological activity of the entrapped biomolecules. Their optical transparency makes them an ideal platform for development of biosensors that rely on transmission of light for detection, such as absorbance or fluorescence measurements. Sol-gel films can be made relatively fast and cast as thin layers on sensor surfaces.

The potential applications of biomolecules entrapped in sol-gel derived materials, particularly in the field of biosensor development, appear to be limitless. One class of biological molecules that has received considerable attention is proteins, with numerous reports having appeared describing the function, structure,²⁹ dynamics,³⁰ accessibility,²⁹⁻³¹ reaction kinetics,³² initial stability,^{2,46} and long-term stability³³⁻³⁴ of entrapped proteins. These studies have established that, in the majority of cases, entrapped biological molecules initially retain at least a portion of their characteristic biochemical functionality, although the variability in function is large, with values between 2%³⁵ and 100%³⁶⁻³⁷ of solution activity having been reported. It has also been found that many biomolecules can remain stable over periods of months.³ However, the extreme pH values and high alcohol levels that are encountered during glass formation can destabilize some less robust proteins.³⁸ Alternatively, positively charged amino acid residues located at or near the active site can interact with the negative silicate matrix, which occurs with lactate and glycolate oxidase.³⁹ In these cases, encapsulation can result in a substantial or even complete loss of function and can cause significant structural changes in proteins initially and over time.⁴⁰ For biosensor development, changes in protein stability with time can lead to changes in sensitivity and consequently drifts in calibration over short time periods.

In light of these results, preliminary studies by Brennan⁴¹ and co-workers demonstrated that durable, optically transparent materials with significant lipase activity can be prepared, optimal materials are produced with TEOS as a precursor and a few weight percent of low molecular weight poly(ethylene glycol) (PEG) as an additive. PVA tended to have a detrimental affect on lipase activity, while PEG provided a concentration-dependent enhancement of the enzyme activity. In general, the physical properties, including transmittance and resistance to cracking, improved with increasing PEG concentration, the inclusion of organic moieties into the matrix affected both the homogeneity of the materials and polarity of the internal environment, with PEG reducing and PVA increasing the internal polarity.

The internal microenvironment of sol-gel derived materials can be probed by using polarity sensitive fluorescent probes 7-azaindole and 6-propionyl-2-(dimethylamino)-naphthalene. Optical absorption spectroscopy is also an important technique to study the interactions between the biomolecule and the sol-gel silica matrix. These interactions are of central importance in determining the synthesis conditions for these materials, in creating the pores which confine the protein, and in stabilizing the protein so that it retain its structure and chemical function. By studying the encapsulated cytochrome c in the sol-gel derived xerogel, Miller and co-workers suggested that cytochrome c resides in pores that conform to its size (32.4 Å diameter). This is curious feature since the gel network possesses a wide distribution of pore sizes, yet the proteins are selectively trapped in pores that conform to the size of the protein (or larger).⁴² Recent studies using resonance Raman spectroscopy provide additional details of this mechanism and further support the templating effect of the dopant biomolecules.

Traditional silica gels are hydrophilic because of the remaining surface silanol groups. The immobilization of lipase, a kind of lipophilic enzyme, in such silica matrix showed a much lower remaining activity (<5 %) than other immobilized enzymes.⁴³ Previous work demonstrated that the matrixes required for achieving high lipase activities consisted of high proportions of alkyl silanes.⁴³ Advances have already made in this direction with recent efforts in sol-gel-based protein entrapment by using different silane precursors, such as organically modified silanes⁴⁴⁻⁴⁷ and co-entrapment of species such as polymers and surfactant along with the protein to modulate material properties and/or protein stability.⁴⁸⁻⁵² Examples include the entrapment of lipase into methyltrimethoxysilane-

based materials, lipase into polymer doped materials formed from organically modified silanes (which showed an 8800% enhancement of activity compared to free lipase for esterification reactions but only 40% for hydrolysis reactions involving emulsified oils),⁴⁵ lipase and human serum albumin (HSA) in organically modified silicates (ORMOSILS), with lipase showing up to 50% activity for the hydrolysis of glyceryl tributyrates,⁴⁶ glucose oxidase and horseradish peroxidase in the presence of a graft copolymer of polyvinylimidazole and polyvinylpyridine,⁵³ and acetylcholinesterase and butyrylcholinesterase in the presence of poly(ethylene glycol).⁵⁴ In each case, the addition of organosilane precursors or polymers resulted in improved function for the entrapped proteins.

Despite the apparent success of the sol-gel techniques, a few factors associated with the current sol-gel immobilization still severely hinder its practical applications in industry. The presence of alcohol as a solvent or a by-product in the sol-gel reactions may have detrimental effect on the structure and properties of entrapped proteins. Further, the apparent activity of an entrapped enzyme is often hindered by internal diffusion resistance of the substrate or analyte, and sometimes, by reduced accessibility in sol-gel matrices, even if extremes of reaction conditions (e.g., pH, temperature, etc.) are avoided to prevent the labile biomolecules from irreversible deactivation. Moreover, the small pore sizes and extensive cross-linking of such materials can lead to problems with capillary stresses which cause the glass to shatter upon immersion into water.⁵⁵ This is obviously a problem for biosensor development, which requires the material to be placed in aqueous biological fluids. In addition, such materials have been shown to have extremely long aging times and a large distribution of internal environments, which leads to time-dependent alterations in entrapped protein function.⁵⁶

So far, many efforts and advancements have been addressed to the above-mentioned problems. Instead using the tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS) precursors, Bhatia⁵⁷ and co-workers developed a novel two-step aqueous route using sodium silicate as the precursor which avoid the liberation of alcohols. Enzymes immobilized in such matrix showed a higher remaining activity compared to that of the immobilized enzymes in TMOS-derived materials. The non-surfactant template pathway⁵⁸⁻⁵⁹ to the mesoporous silica materials developed in Dr. Wei's group also proved to be successful in the entrapment of some robust enzymes like alkaline phosphatase, acid phosphatase, horseradish peroxidase and glucose oxidase.⁶⁰⁻⁶⁴ The immobilized enzymes showed reasonable remaining activity, and much higher thermal stability.

Organophosphorus compounds (OPs) are highly toxic and found extensive use as pesticides, insecticides and potential chemical warfare (CW) agents. Recently, because of environmental, health and national security concerns, significant efforts have been directed towards developing sensitive and portable sensors for these compounds. According to the definition of the International Union of Biochemistry, enzymes catalyzing the hydrolysis of P-F or P-CN bonds in organophosphorus compounds are classified as organophosphorus acid anhydrolases (OPAA; EC 3.1.8.2).⁶⁵ The OPAA enzymes, from a genetically altered *E. Coli* bacterium, are quite unusual and capable of catalytically hydrolyzing a wide variety of organophosphorus compounds including a commonly used substrate, the serine protease inhibitor diisopropyl fluorophosphates (DFP), and different fluoride-containing G-type nerve agents such as soman (GD, *O*-pinacolymethylphosphonofluoridate), sarin (GB, *O*-isopropylmethylphosphonofluoridate), GF (*O*-cyclohexyl methylphosphonofluoridate), and cyanide-containing tabun, GA (ethyl N, N-dimethylphosphoramidocyanidate).⁶⁶⁻⁶⁷ Although little is understood about the native substrate or function, such enzymes have considerable potential for decontamination of toxic OPs and got a boost with the advancement on the identification, purification and large-scale production of OPAA.⁶⁸⁻⁶⁹

The currently fielded decontamination solution, DS2 and bleach are quite effective in decontaminating the CW agents. However, they are toxic and corrosive in nature that makes them hazardous for use by personnel, on equipment and for environment. It is also a logistical burden due to the weight and volume of the product and packaging. The enzyme-based decontamination system not only provides rapid removal of CW agents, but is also safe for users and benign on the environment. Cheng⁷⁰ and co-workers developed a simple and safe enzyme-based decontamination system, where the lyophilized OPAA enzyme is mixed into spray or foam with the existing spray or fire-fighting equipment. Such a system can be employed for CW decontamination in large areas such as equipment, vehicles, port facilities, stockpiles, large fixed sites, and clean-up operations that might result from a terrorist incident. Another approach is the application of OPAA in skin lotion or cream that also provides opportunity for personnel protection.

However, there are still many formidable obstacles in the practical applications of these enzymes. Among the problems is that the enzymes, like many typical proteins, are relatively unstable under harsh conditions. For example, the enzymes lose their activity easily in the presence of organic solvents, at elevated temperature (e.g. > 40-60 °C) or over long period of storage because of protein denaturation. Furthermore, unprotected enzymes are vulnerable to the attacks from other biological substances such as proteases. In light of this, the development of a safe, long-term storage and non-corrosive OPAA-based decontamination system is highly desired. Moreover, enzymes would be more widely used in organic solvents if their activity were not so drastically reduced. All of these problems can be solved by immobilizing the enzymes in appropriate matrix with tunable porosity and functionality.

The ability to successfully manipulate the properties of sol-gel derived bioglass obviously requires a detailed understanding of the development of the materials. In the present study we have aimed to understand the effects of organic functionality on the physicochemical properties of sol-gel derived materials and attempted to correlate this to enzyme activity by encapsulating the biocatalyst OPAA into mesoporous silica host materials of various compositions. OPAA was chosen owing to its ability to catalyze the hydrolysis of highly toxic organophosphorus compounds, which has potential application as the enzyme-based decontaminant system in the biological and chemical warfare. The organic functionality was incorporated into the matrix via the co-condensation of TMOS and MTMS and PTMS. The immobilization is achieved by nonsurfactant-templated sol-gel method recently developed in our lab. The immobilization conditions, such as pH, template type and concentration, organic functionalization were investigated to achieve high remaining activity of the immobilized OPAA. The stability of the immobilized OPAA in the organic solvent, methanol, was also investigated and correlated with the microstructure of the silica matrix. The material properties that were examined included optical clarity (which is important in the development of optical sensors), pore structure (surface area, pore volume, pore size distribution), and hydrophilicity/hydrophobicity.

2.0. Experimental Section

2.1. Materials

Tetramethyl orthosilicate (TMOS, 99 %), methyltrimethoxysilane (MTMS, 97 %), propyltrimethoxysilane (PTMS), were obtained from Aldrich Chemical Co. (Milwaukee, WI). D(-)-fructose, poly(ethylene glycol) (PEG, MW 400), diisopropyl fluorophosphate (DFP), isopropanol,

Bis-Tris Propane (BTP) were purchased from Sigma Chemicals (St. Louis, MO), HCl was product of Fisher Scientific (Fair Lawn, NJ). Organophosphorus acid anhydrolases (OPAA, EC 3.1.8.2) were provided by Dr. Tu-Chen Cheng from U.S Army (SBCCOM) in the form of pH 7.3 BTP buffer solution (6.5 mg/ml). Sodium fluoride is the product of Orion. All chemicals and reagents were used as received without further purification.

2.2. Preparation of OPAA-Containing Biogels

The OPAA was immobilized directly in the mesoporous silica materials via the nonsurfactant-templated pathway developed in our group. It was implemented by two different approaches. In the first approach, the nonsurfactant compound, fructose, was employed as the template during the immobilization of OPAA, both TMOS and mixture of TMOS/MTMS (molar ratio 3:1) were used as the precursors to form the pure silica and organically modified silica matrices, respectively. The OPAA-containing buffer solution was added to the pre-hydrolyzed precursors before gelation. This procedure was proven successful in cases of HRP and GOx as described in detail in chapter 4 and chapter 5, respectively. In the second approach, in place of fructose, another organic compound, poly(ethylene glycol) (PEG, MW 400) was employed as the template to accelerate the gelation for fast immobilization of enzyme, especially in the cases where higher content of MTMS in the precursor mixture was used (up to 75 mol%). In order to minimize the deleterious effect of the liberated alcohol (methanol in case of TMOS) on the enzyme, the acid-hydrolyzed precursors were first degassed under vacuum to remove most of the water and alcohol. The pH of the mixture was tuned to around neutral by adding BTP buffer solution. Template was introduced to the above mixture followed by the addition of buffered enzyme solution. The mixtures were quickly mixed, sealed with Paraffin film and let it stand at room temperature until gelation occurred. After gelation, 15 pin-holes were punched on the Paraffin film to let the evaporation of the solvent and by-product (methanol in this case). All the samples were aged in the hood overnight and then put in vacuum oven for three days to accelerate drying process. The samples were crushed into powders and stored in the refrigerator for further characterization.

As a typical OPAA-containing sample synthesized from the TMOS and 25 mol% MTMS in the presence of 50 wt% of fructose (denoted as OPAA-HF50), 2.320 g TMOS (15.0mmol) and 0.690 g MTMS (5.0mmol) were mixed with 0.70 g distilled water and 30 μ L 40mM HCl (1.2×10^{-2} mmol) under magnetic stir at room temperature. After \sim 15 minutes, the precursors mixture became homogeneous, accompanied by the raise of temperature. Upon cooling to room temperature, 1.260 g fructose dissolved in 1.260 g distilled water (50 wt% solution, which account theoretically for 50 wt% in the final dried hybrid silica materials) was added to the pre-hydrolyzed TMOS/MTMS sol under agitation. Upon cooling the mixture to \sim 0 $^{\circ}$ C in dry ice bath, 1 ml BTP buffer (50 mM, pH 7.3) containing 0.15 mg OPAA was added. The resultant homogeneous sol was sealed with paraffin film, which had 15 pinholes punched with a syringe needle to allow the evaporation of volatile molecules such as methanol and water. Gelation occurred within few minutes. After drying in air overnight then in vacuum to reach constant weight at room temperature, a transparent, monolithic disk of sample was obtained. The detailed composition of the samples (OPAA-F series and OPAA-HF series) made in this approach was listed in Table 1.

A typical OPAA-containing sample synthesized by the low-shrinkage approach from pure TMOS as precursor in the presence of 50 wt% of PEG, denoted as OPAA-TMOS-PEG50, 3.120 g TMOS (20.0 mmol) was mixed with 0.7 g distilled water and 30 μ L 40mM HCl (1.2×10^{-2} mmol) under magnetic stir at room temperature. After \sim 30 minutes, the pre-hydrolyzed sol was connected to a vacuum pump (1 mmHg) to remove the solvent water and by-product methanol. The water and

methanol were collected in cold-trap which was immersed in the mixture of dry ice and acetone. The occurrence of the bubbles slows until it takes several minutes for a single bubble form. After continuing degas for about 1 h, the volume of the sol shrank to ~ 50 % of its initial volume. To this degassed sol, 1.26 g PEG (MW 400) was added drop by drop under constant stir, 0.5 ml BTP buffer solution (50 mM, pH 7.3) was then added to adjust the pH of the mixture above 6.0 (which is favorable for the entrapment of enzyme). Gelation occurred within a minute after the addition of 0.5 ml OPAA buffer solution (50 mM, pH 7.3 BTP buffer, containing 0.30 mg OPAA). The mixture was sealed with Paraffin film, which had ~ 15 pinholes punched by a syringe needle to allow the evaporation of the solvent and by-product of methanol. After drying in air overnight and then in vacuum oven for three days to accelerate the drying process, a transparent, millimeter sized disk sample was obtained. The detailed composition of the samples (OPAA-TMOS-PEG50, OPAA-TMOS-F50, OPAA-TMOS-Cont, OPAA-MTMS-PEG50, OPAA-MTMS-Cont, OPAA-3MTMS-PEG50, OPAA-3MTMS-Cont) made in this approach was listed in Table 2.

2.3. Characterization of the OPAA-Containing Biogels

The template was extracted from as-synthesized samples to yield mesoporous OPAA-containing biogels. The detailed extraction procedure can be found in the experimental section of chapter 4. Infrared spectra of the as-synthesized, buffer solution extracted samples were measured in the form of KBr powder-pressed pellets on a Perkin Elmer 1600 FT-IR spectrophotometer (Norwalk, CT) with a resolution of 4 cm^{-1} . The BET (Brunauer-Emmett-Teller) surface areas of OPAA-containing biogels after removing template were characterized by N_2 adsorption-desorption isotherms conducted on a Micromeritics ASAP (Accelerated Surface Area and Porosimetry) 2010 Instrument (Micromeritics, Inc., Norcross, GA) at -196°C . Prior to the measurements, the samples were degassed at 120°C and 1 Pa overnight. The BJH⁷¹ models were used to obtain the pore size distribution (PSD) and pore volume of the gels.

2.4. Activity Assay of Free and Immobilized OPAA

OPAA can catalyze the hydrolysis of a wide variety of organophosphorus compounds, in this experiment, diisopropyl fluorophosphates (DFP), a less toxic analog of the organophosphonate agents, were used as the substrate/simulant to characterize the activity of free and immobilized OPAA.⁷⁰ pH/ion meter model 450 (Corning), combination fluoride ion selective electrode (Orion) were used to monitor the concentration change of fluoride ion which is generated during the OPAA catalyzed hydrolysis of DFP. The fluoride ion selective electrode is first calibrated with standard sodium fluoride solution. Spontaneous DFP hydrolysis was determined by measuring the fluoride ion concentration change during DFP hydrolysis alone in 2.475 mL BTP buffer (50 mM, pH 8.0) and 2 μL 100 mM MnCl_2 without adding the enzyme OPAA. Once the spontaneous rate of DFP is determined, a fresh BTP buffer solution (2.475 mL, 50 mM BTP at pH 8.0) and 2 μL 100 mM MnCl_2 were added to the reaction vessel with a dilute concentration of OPAA. Measurement started as soon as 25 μL of DFP was added to the reaction vessel at interval of two minutes for total ten minutes, the DFP hydrolysis rate was calculated using the highest linear slope obtained from the second and fourth point of measurement. The enzyme activity was assessed from the difference of the DFP spontaneous rate and OPAA-catalyzed DFP hydrolysis rate. For free OPAA, the stock solution (15.0 mg/ml) was dilute with BTP buffer (50 mM, pH 8.0) at the ratio of 1:50 and then 2 μL (which contain 2.5 μg OPAA) was used directly. For immobilized OPAA samples, certain amount of powder samples (which contain 10 μg OPAA) were weighed and extracted with dilute BTP buffer (50 mM, pH 7.3) solution three times before the activity assay. For detailed extraction procedure,

please refer to the experimental section of chapter 4. All measurements were made in triplicate to obtain the average values.

2.5. Stability of the Free and Immobilized OPAA in Organic Solvent

To test the effect of the organic solvent on both free and immobilized OPAA, the OPAA were first soaked in 20 % (v/v) methanol solution and then the activity assay was performed as described above. The remaining activity of free and immobilized OPAA over time were assayed at several time intervals and compared.

3.0. Results and Discussion

3.1 Nonsurfactant-Templated Sol-Gel Immobilization of OPAA

Biological species are very sensitive to alcohol and pH. The usual sol-gel procedure has then been to be slightly modified to meet these requirements. The neat alkoxide is pre-hydrolyzed under acid-hydrolyzed conditions before biomolecules are added to the solution. Methanol is released during this hydrolysis reaction. It behaves as a co-solvent, and a transparent solution of hydrolyzed precursors is obtained after a few minutes accompanied with temperature increase because of the exothermic hydrolysis. After cooling to room temperature, this solution is then mixed with a buffered solution (pH~7) containing the biomolecules. Condensation reactions are quite fast at this pH, and a gel network rapidly grows in which the biomolecules remain embedded. Some enzymes are quite fragile and in these cases entrapment has to be performed at around 0 °C. Without the template or pore forming agents, the thus synthesized xerogels (air-dried gel) are microporous with pore size typically around 10 Å. In the presence of the template, the pore parameters can be tuned to some extent simply by changing the concentration of the template during the sol-gel process, which is demonstrated in detail in previous chapters.

3.2. Low-Shrinkage Sol-Gel Immobilization of OPAA

In traditional and most of the modified sol-gel immobilization, sol-gel matrices have been prepared by hydrolysis and condensation of an orthosilicate such as TMOS or TEOS. First, TMOS (TEOS) is partially hydrolyzed in an acidic medium by addition of controlled amount of water. Next, the biological species are introduced in a suitable buffer solution to facilitate gelation. The pH of the buffer solution is chosen to allow the final solution to be close to neutrality in order to avoid denaturation of proteins. However, hydrolysis of TMOS (or TEOS) as starting material leads to generation of methanol (or ethanol), presence of which in large quantities can be deleterious to proteins and cells. In low temperature aging typically used with encapsulation of biological species, the generation of alcohol proceeds for an extended period of time allowing the encapsulated species to denature over time. Moreover, this process involves considerable shrinkage from wet gel into xerogel (normally 1/8 of its original volume), thus exerting pressure on the encapsulated enzymes.

In light of this, we adapted the low-shrinking sol-gel procedure⁷² which was developed in our lab for the immobilization of the OPAA. This procedure has several advantages over the traditional sol-gel encapsulation. First, most of the alcohol liberated during the hydrolysis is removed before the addition of OPAA, this minimizes the denaturation of the OPAA during the immobilization. Second, removal of the alcohol and water reduce the large-scale shrinkage and pore collapse during the xerogel formation. The template molecules added also help the pore forming and preserve the pore

structure. In our experiment, the volume shrinkage of the OPAA-containing samples is around 1/2 of their original volume. Third, the removal of the solvent from the sol accelerates the gelation, which is favorable for the immobilization of enzyme since the enzyme molecules can be frozen in its active conformation within the silica matrix. Prolonged gelation time will result in the denaturation of the enzyme in the presence of alcohol over time.^{8,12}

3.3. Surface Area and Pore Structure of the Silica Matrix

The surface area and pore structure of the silica matrix synthesized from the TMOS and TMOS/MTMS in the presence of nonsurfactant compounds, fructose in this case, has been characterized by N₂ adsorption-desorption isotherms as discussed in detail in chapter 2. Since the amount of OPAA is fairly small (~ 0.3 mg) compared to that of the template molecules, the entrapment of the OPAA in such silica materials does not change the structure of the silica matrix. Figures 1, and 3 show the representative N₂ adsorption-desorption isotherms for the water-extracted samples synthesized from fructose-templated pure TMOS and TMOS/MTMS (molar ratio 3:1), respectively. As the concentration of fructose increases from 0 to 60 wt%, the isotherms changes gradually from reversible Type I, typical of microporous materials, to Type IV with H2 hysteresis loop, typical of mesoporous materials. The incorporation of the methyl group into the silica matrix (OPAA-HF series samples in Table 1) via the nonhydrolyzable Si-C covalent bond increase the hydrophobicity but decrease the pore volume and pore size. The BJH pore size distribution calculated from the desorption branch of the isotherm showed the peak pore diameter around 3.5-5.0 nm for pure TMOS-derived samples (Figure 2) and 3.0-4.0 nm for the TMOS/MTMS-derived samples (Figure 4).

Figures 5 and 6 are the N₂ adsorption-desorption isotherm and BJH pore size distribution, respectively, for the OPAA-containing samples synthesized in the presence of 50 wt% PEG as template. TMOS-PEG50-OPAA is a typically mesoporous silica material with pore size around 3.2 nm, while sample MTMS-PEG50-OPAA is predominantly microporous material. The introduction of the methyl groups into the silica matrix increase the hydrophobicity but decrease the pore volume, 0.71 cm³g⁻¹ for TMOS-PEG50-OPAA and 0.61 cm³g⁻¹ for MTMS-PEG50-OPAA.

As for the mechanism of the mesopore formation during the template sol-gel immobilization, we speculate that both of the pore forming agent and biomolecules participate in the mesophase formation. In this way, the dopant biomolecules serves as a nucleus which enables condensation polymerization to readily take place. Thus, the protein acts as a structural template around which the gel network can develop and form a porous inorganic polymer cage.

The infrared spectrum of both as-synthesized and after-extracted OPAA-containing samples prepared in the presence of 50 wt% fructose and PEG400 are shown in Figures 7 and 8, respectively. The as-synthesized samples shows major absorption bands associated with fructose and PEG molecules. For example: symmetrical and asymmetrical -CH₂ stretching occur at 2852 and 2922 cm⁻¹ respectively, and C-C-O stretching vibration designated at ~1100 cm⁻¹. These bands disappeared in the water-extracted samples, indicating a complete removal of the template molecules.

3.4. Dependence of OPAA Activity on the Silica Pore Structure

The as-synthesized OPAA-containing samples were first extracted with BTP buffer (50 mM and pH 7.3) before doing the activity assay. It is well known that the activity of immobilized enzymes are highly depended on the pore structure, since the diffusion of the substrate into and

product out of the silica matrix play an important role during the enzymatic activity measurement. OPAA immobilized in the microporous silica matrix without adding template, i.e. TMOS-cont-OPAA and MTMS-cont-OPAA and 3MTMS-cont-OPAA, show little or no apparent activity. The introduction of the template, especially when the template concentration is above 50 wt%, significantly improve the accessibility of the enzyme OPAA to the substrate DFP. In these materials, there are numerous interconnected mesopores or channels which can entrap the OPAA efficiently without leakage and facilitate the diffusion of the small molecules (both substrate and products) in and out of the silica matrix.

Our preliminary results showed that the OPAA immobilized in pure and low content (25 mol% MTMS) of organic-modified silica matrices synthesized via the first approach has low remaining activity, i.e. < 10 % compared with the free OPAA in solution (Figure 9). This may due to several factors, including enzyme denaturation during the sol-gel process because of the presence of the liberated methanol, reduced pore size arise from the introduction of organic groups onto the surface of the silica matrix and low affinity of substrate to the enzymes.

The presence of MTMS results in an improvement in protein function which becomes greater as the level of MTMS increases (Figure 10), and provides activity values that are ~2 fold higher than those obtained in TMOS-derived samples. Take the pore size of the TMOS and MTMS/TMOS(1:1 molar ratio)-derived silica matrix into account as shown in Figure 6, even the pore size of methyl-modified silica matrix is much less than that of the pure silica matrix, the OPAA immobilized in methyl-modified silica matrix has higher enzymatic activity than that in pure silica matrix. The presence of PTMS resulted in no improvement in activity. This result was somewhat unexpected, and may be due to precipitation/denaturation of the OPAA molecules by the liberated methanol during the prolonged sol-gel process, since the gelation time of PTMS-derived sample is much longer than those of TMOS- or TMOS/MTMS-derived samples.

At high level of MTMS, the incorporation of the methyl group into the silica matrix significantly improves the microenvironment of the immobilized enzyme, and assist in the solubilization and even dispersion of the oily organophosphorus compounds for enzyme action.

Besides the commonly used the nonsurfactant templates, like fructose or glucose, we also tried to employ the use of nonionic polyethylene glycol (PEG, MW 400) as the template during the sol-gel immobilization of OPAA. This idea comes from several considerations. First, PEG has long been known as protectant to preserve the overall enzyme structure and minimize enzyme inactivation during the lyophilization.⁷³ Second, at the higher concentration of organosiloxane, the fructose will precipitate from the hydrolyzed sol and resulted inhomogeneous, opaque sample, while for PEG, it will form homogeneous sol before gelation. Third, the addition of PEG accelerates the gelation and preserves the transparency of the samples even under high concentration.⁶³ Fourth, previous research has demonstrated that the use of PEG has no deleterious effect on the physical properties of the sol-gel materials.³

3.5. Stability of Immobilized OPAA in Organic Solvent

Although enzymes are very efficient and specific in catalyzing certain kinds of reaction, they also have some drawbacks in their practical applications. Among the problems is that the enzymes, like many typical proteins, are relatively fragile and unstable under harsh conditions including elevated temperature, severe pH and presence of organic solvents. Furthermore, unprotected enzymes are vulnerable to the attacks from other biological substances such as proteases.

Our results show that the activity of free OPAA decreased sharply when mixed with methanol, i.e. 50 % drop in activity after soaking in 20 % (v/v) methanol solution for just 10 minutes. The decreased enzymatic activity was also demonstrated by the immobilized OPAA synthesized by the first approach in our experiment (< 10% remaining activity than that of free OPAA in solution), where the denaturation of OPAA molecules in the liberated methanol during the hydrolysis of the precursors can be attributed partly to the low remaining OPAA activity. This prompted us to use the low-shrinking sol-gel approach developed in our lab in the sol-gel immobilization of OPAA. This low-shrinking approach minimized the denaturation of the OPAA and proved highly effective in preserving the OPAA activity (Figure 11). Compared to the OPAA-containing sample synthesized in the presence of 60 wt% of fructose via the first approach, sample synthesized from TMOS in the presence of 50 wt% of fructose or PEG via the second approach showed almost ~ 15 fold increase in the activity. The sample in the presence of PEG as template has slightly higher activity than that in the presence of fructose as template, which can be attributed partly to the stabilizing effect of PEG on OPAA during the sol-gel process.

The encapsulation of the OPAA in the silica matrix leads to stabilizing effects. First, protein denaturation due to methanol is reversible in the confined sol-gel matrix, where the OPAA molecules remain in the folded state as compared to the unfolded (denatured form) in the solution. Immersing the methanol-soaked samples in pure buffer led once again to the native OPAA. A second significant feature of sol-gel immobilization is that protein aggregation did not occur, even when the gels were soaked in methanol solution several days, a condition which produces protein aggregation in solution. These results suggest that confining the OPAA molecules within the mesoporous silica matrix not only avoid leakage but also constrain the long-range migration of the protein so that the aggregation is prevented, yet the molecules remain sensitive to changes in the local environment. Further investigation of the protein folding-unfolding in rigid matrix artificial chaperone⁷⁴⁻⁰ (RMAC) is currently underway in our lab.

Figure 12 illustrated the activity of free and immobilized OPAA soaked in 20 % (v/v) methanol. For free OPAA, the time difference is 10 minutes, while for the immobilized OPAA, the measurement was taken at intervals of 24 h for three days. The free OPAA showed a sharp decrease of activity of 50 % after soaking in 20% methanol for just 10 minutes, while for the immobilized OPAA, after soaking in methanol for 24 h and then back in BTP buffer solution, the activity was retained or even higher than that before methanol treatment. The increased activity can be attributed to two factors: (1) further removal of the template from the mesopores and the reduced diffusion of the substrate within the silica matrix; (2) constrained cage effect avoid the irreversible denaturation of the immobilized OPAA molecules. The drop in the activity in third day might arise from the fact that the activity assay was conducted in relatively high pH (8.0-8.5), under this pH, hydroxide ions (OH-) can attack and dissolve the silica matrix, which cause the collapse of the pore structure and catastrophic loss of entrapment efficiency. It has been known that silica-based supports are not stable in an alkaline medium, and even prolonged use in neutral pH aqueous solutions can lead to the leakage of immobilized dopant from the supports.¹

4.0. Conclusions

The enzyme OPAA has been successfully entrapped in the mesoporous silica (both pure and organically modified hybrid) matrices in the presence of nonsurfactant template, fructose and PEG via the modified low-shrinking method. Removal of the methanol from hydrolyzed sol before the addition of the OPAA-containing buffer solution not only prevents the denaturation of the proteins

but also reduces the volume shrinkage of the silica matrix. These results define a strategy for the encapsulation of the biomolecules within the sol-gel matrix. The pH/buffer/alcohol stability of the protein in the mesoporous silica matrix provides an important guideline for successful sol-gel synthesis as well as for identifying optimum synthetic conditions for protein function. Finally, it was shown that encapsulation of OPAA within the sol-gel matrix provides stabilization towards external reagents like the organic solvent. Once entrapped within the network, denaturation of the biomolecules is reversible and the native form can be regenerated. In addition, protein aggregation is avoided because of the restriction in protein mobility.

Overall, the ability to entrap OPAA into optically transparent hybrid mesoporous materials with up to 30 % retention of solution activity and enhanced stability in the organic solvent indicates that the development of enzyme-based decontaminant and optical biosensor based on these biomaterials is highly feasible. In fact, investigation is in active progress in our laboratories to explore these nanobiomaterials for practical applications in decontamination creams/lotions and as decontaminants under battlefield conditions. In addition we are carrying out basic studies, both theoretically and experimentally, on several fundamental aspects of OPAA, such as its structure, and folding/unfolding thermodynamics and kinetics both in solutions and in its encapsulated form. The concept established in the present study should be general and readily extendable to many other civilian and military applications including biosensors, biocatalysis, pharmaceuticals, bioreactors, removal of environmental pollutants, etc.

5.0. Acknowledgments

The report has been resulted from a collaborative research between Drexel University, US Army Research Laboratory and Edgewood Chemical Biological Center. The Drexel University team includes Dr. Yen Wei, Principal Investigator on this project, Dr. Hua Dong and Mr. Zhengfei Sun. The Army team includes Dr. Ray Yin, Ms. Kate Ong and Dr. Tu-Chen Cheng. The text in this report is mostly adopted from Dr. Hua Dong's Ph.D. dissertation (Chapter 6, Drexel University, March 2002). It is noted that Ms. Ong is also a doctoral candidate at Drexel University working under the supervision of both Dr. Wei and Dr. Yin, who holds a Visiting Professor appointment at Drexel University. We are most grateful to Dr. Stephen Lee of ARO for his guidance, assistance and support throughout the project. We thank Drexel University for supporting this project by providing teaching assistantship to Hua Dong. The work has been performed under ARO Research Agreement No. DAAD19-01-1-0685 from July 1, 2001 to December 31, 2001.

Table 1. Composition and Pore Parameters of Water-Extracted OPAA-containing Mesoporous Silica Matrices Synthesized from the First Approach

Sample ID ^a	D-fructose ^b (wt%)	S _{BET} ^c (m ² .g ⁻¹)	V _{SP} ^d (cm ³ g ⁻¹)	D _{BET} ^e (Å)	micropore ^f	
					area (m ² .g ⁻¹)	vol. (cm ³ .g ⁻¹)
OPAA-Cont	0	465	0.270	24.1	168	0.094
OPAA-F0	0	449	0.254	23.0	145	0.080
OPAA-F20	20	573	0.320	22.6	140	0.079
OPAA-F40	40	635	0.515	31.5	--	--
OPAA-F50	50	717	0.730	39.5	--	--
OPAA-F60	60	741	0.985	51.7	--	--
OPAA-H-Cont	0	557	0.318	23.4	188	0.110
OPAA-HF0	0	541	0.311	23.0	124	0.130
OPAA-HF20	20	730	0.413	22.9	191	0.110
OPAA-HF40	40	838	0.624	28.8	--	--
OPAA-HF50	50	776	0.673	33.3	--	--
OPAA-HF60	60	786	0.837	41.1	--	--

^a First series samples starting with OPAA-F were synthesized from the pure TMOS as precursors, second series samples starting with OPAA-HF were synthesized from the mixture of TMOS/MTMS (molar ratio of 3:1), control samples were made from the precursors in the presence of 1 mL buffer solution without adding the OPAA and the template, fructose. ^b Theoretical value from the feeding composition of the reaction mixture. ^c The BET surface area using the Kelvin equation in the relative pressure (P/P₀) of 0.10-0.30. ^d The single point pore volume determined at P/P₀≈1. ^e Determined by the t-plot method. ^f The BET pore diameter calculated from 4V/S.

Table 2. Compositions of Low-Shrinkage OPAA-containing Mesoporous Silica Materials

Sample ID	Precursor(s) (molar ratio)	Template	Mass (g)	Appearance
OPAA-TMOS-C	TMOS	No	1.3967	Transparent
OPAA-TMOS-F50	TMOS	Fructose	2.8242	Transparent
OPAA-TMOS-PEG50	TMOS	PEG	2.7518	Transparent
OPAA-MTMS-C	TMOS/MTMS (1:1)	No	1.3593	Transparent
OPAA-MTMS-PEG50	TMOS/MTMS (1:1)	PEG	2.6000	Transparent
OPAA-3MTMS-C	TMOS/MTMS (1:3)	No	1.3797	Translucent
OPAA-3MTMS-F50	TMOS/MTMS (1:3)	Fructose	2.6030	Opaque
OPAA-3MTMS-PEG50	TMOS/MTMS (1:3)	PEG	hydrogel	Translucent

Note: The loading of the OPAA in each sample is 0.3 mg, since the sol is homogeneous before gelation, OPAA is evenly distributed in the whole sample.

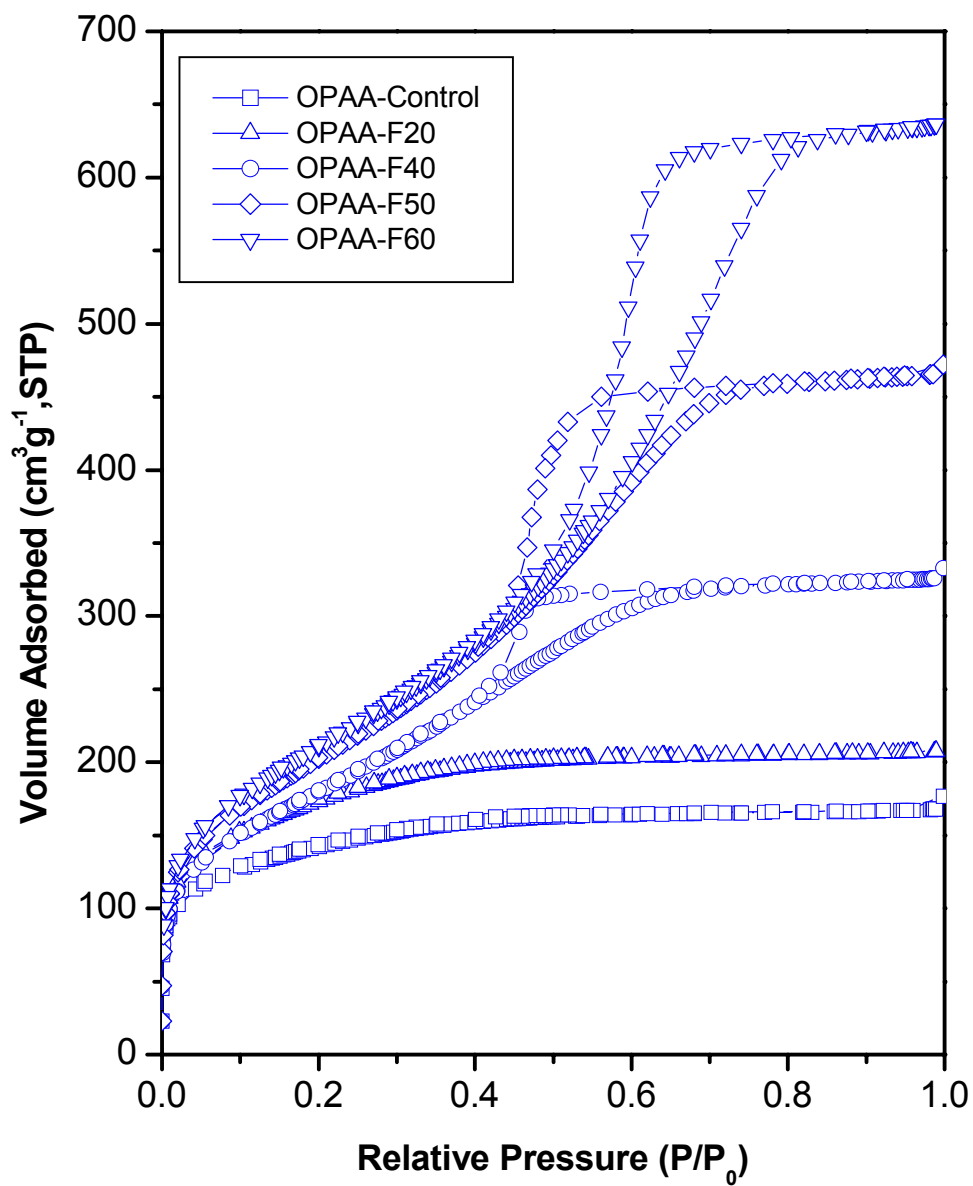


Figure 1. N₂ adsorption-desorption isotherms at -196 °C for the water-extracted OPAA-containing sol-gel matrices synthesized in the presence of 0-60 wt % D-fructose.

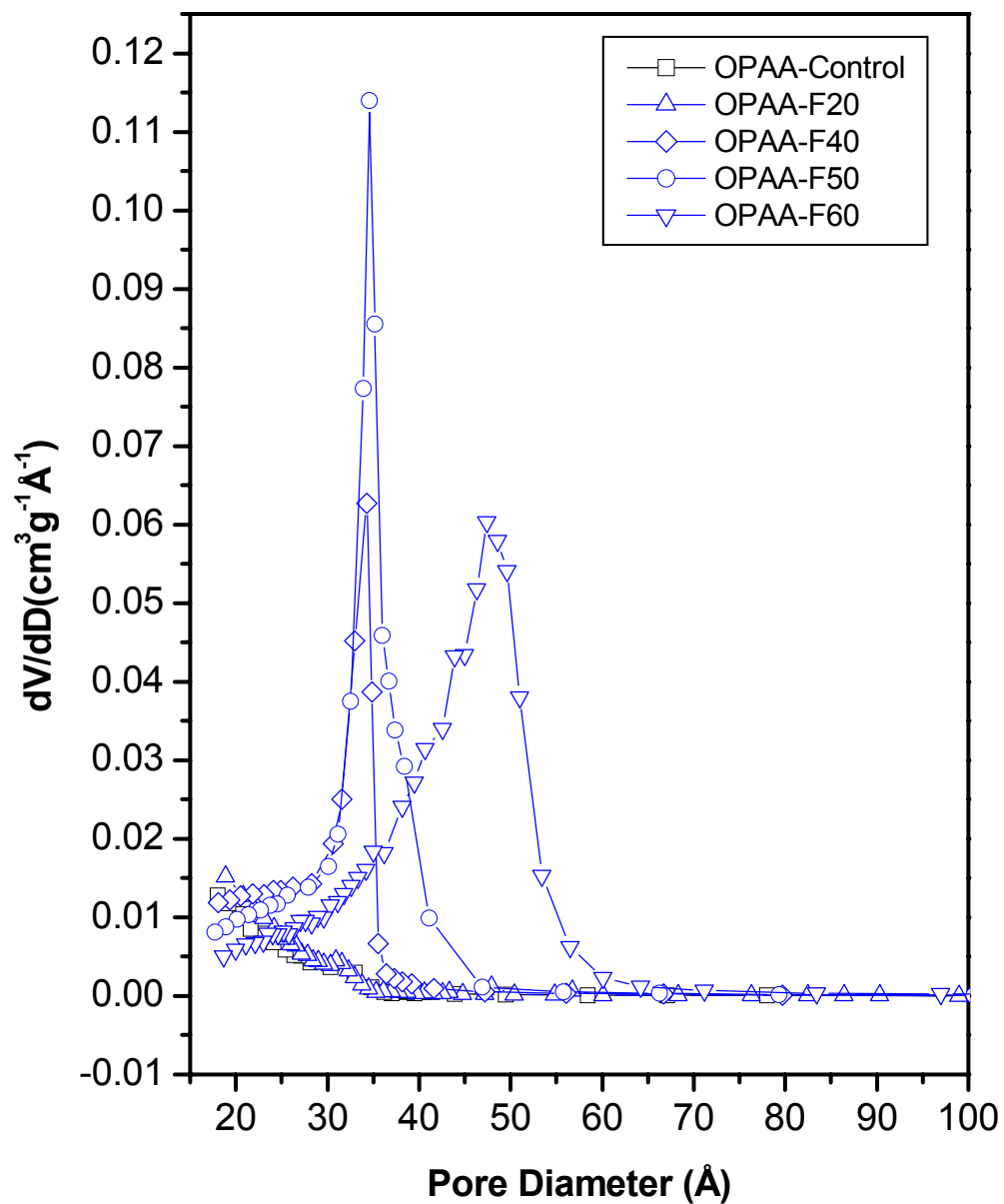


Figure 2 The BJH desorption pore size distributions from the N₂ desorption isotherms for the OPAA-containing hybrid sol-gel samples synthesized in the presence of 0-60 wt % D-fructose.

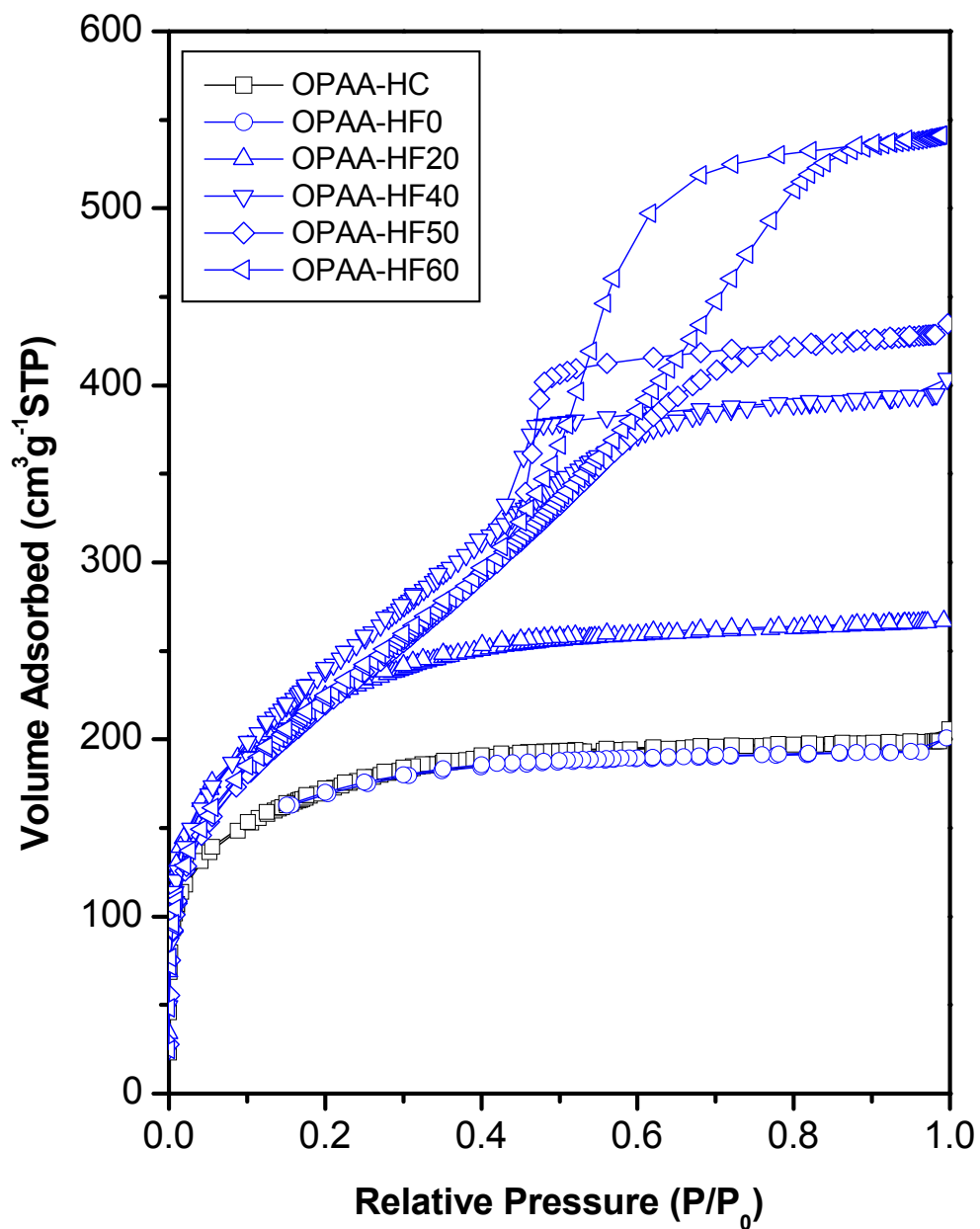


Figure 3. N₂ adsorption-desorption isotherms at -196 °C for the water-extracted OPAA-containing hybrid sol-gel matrices synthesized in the presence of 0-60 wt % D-fructose. Precursors is composed of 75 mol% TMOS and 25 mol% MTMS.

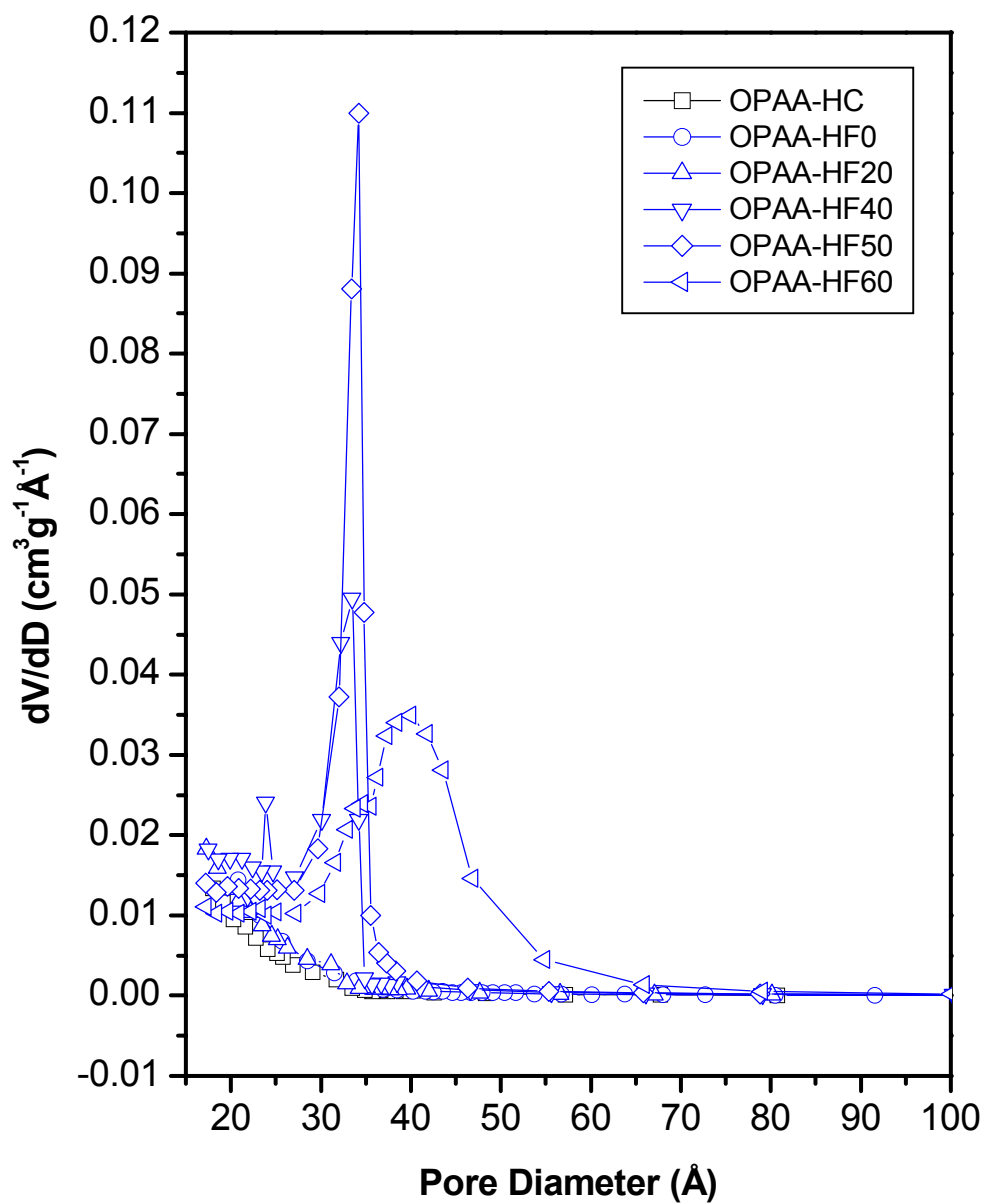


Figure 4. The BJH desorption pore size distributions from the N₂ desorption isotherms for the OPAA-containing hybrid sol-gel samples synthesized in the presence of 0-60 wt % D-fructose. The precursors composed of 75 mol% TMOS and 25 mol% MTMS.

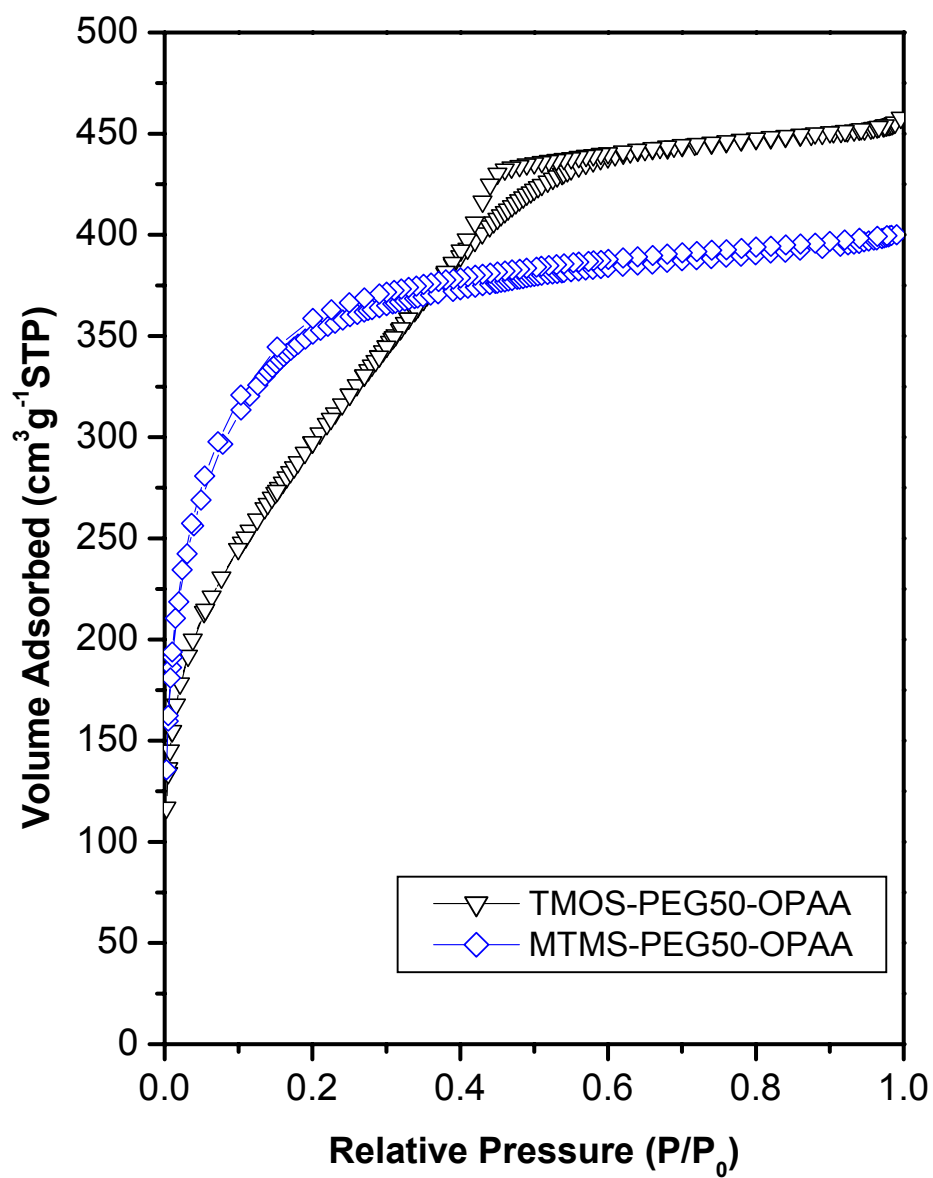


Figure 5. N₂ adsorption-desorption isotherms at -196 °C for the water-extracted OPAA-containing sol-gel matrices synthesized in the presence of 50 wt % PEG (MW 400).

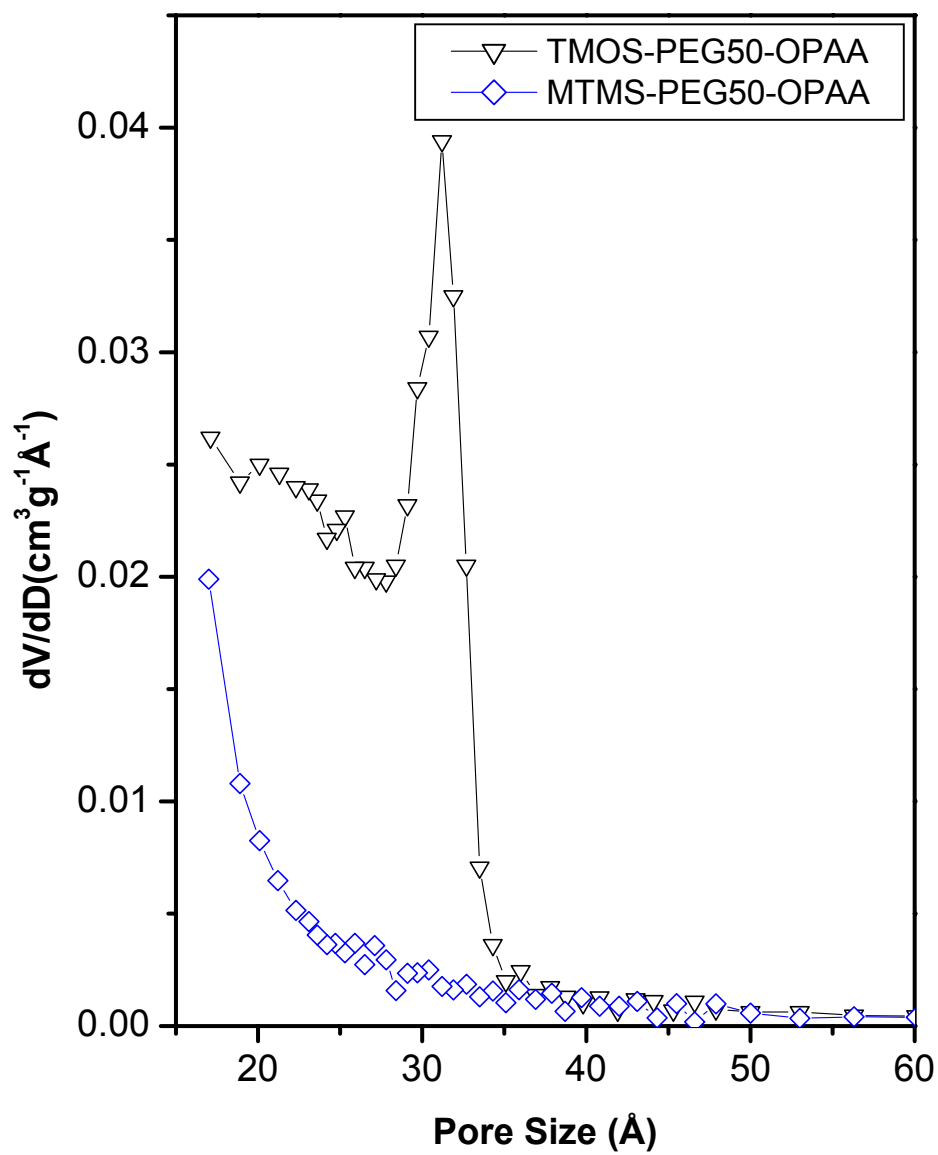


Figure 6. The BJH desorption pore size distributions from the N₂ desorption isotherms for the OPAA-containing hybrid sol-gel samples synthesized in the presence of 50 wt % PEG (MW 400).

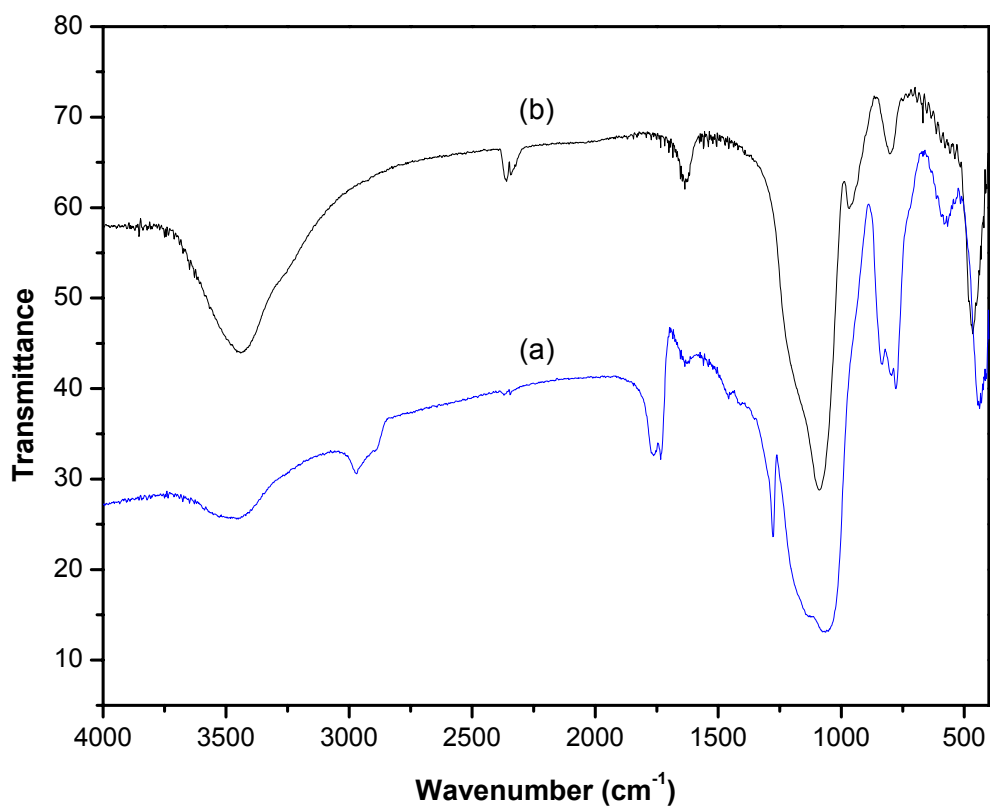


Figure 7. FT-IR of OPAA-containing samples synthesized from the hydrolysis of TMOS in the presence of 50 wt% fructose as template. (a) before water extraction (b) after water extraction.

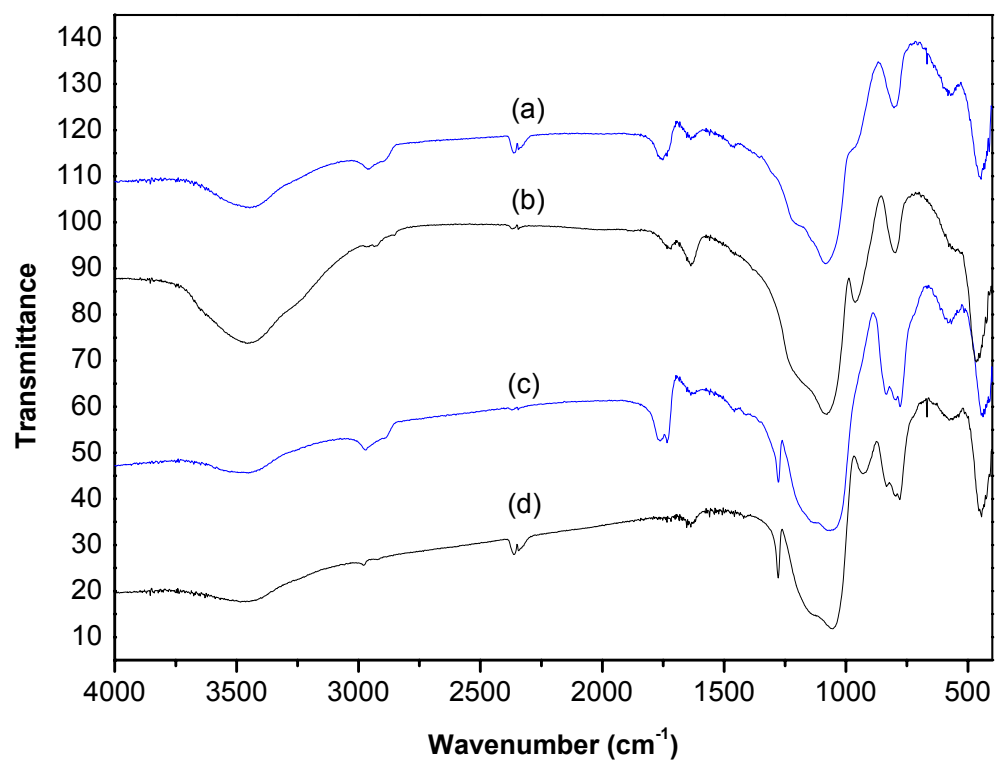


Figure 8. FT-IR of OPAA-containing samples synthesized from the hydrolysis of TMOS or TMOS/MTMS(1:1) in the presence of 50 wt% PEG as template. (a) TMOS, before water extraction (b) TMOS, after water extraction (c) TMOS/MTMS, before extraction (d) TMOS/MTMS, after extraction.

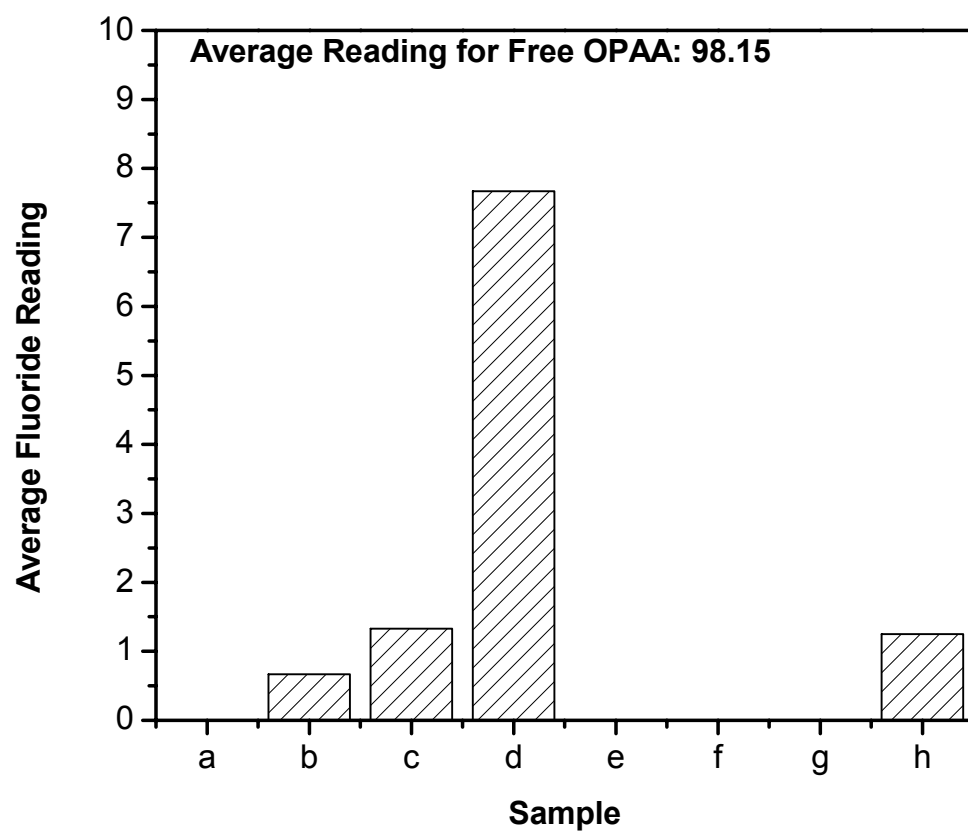


Figure 9. Activity of immobilized OPAA via the first approach (a) OPAA-F0, (b) OPAA-F20, (c) OPAA-F40, (d) OPAA-F60, (e) OPAA-HF0, (f) OPAA-HF20, (g) OPAA-HF40, (h) OPAA-HF60.

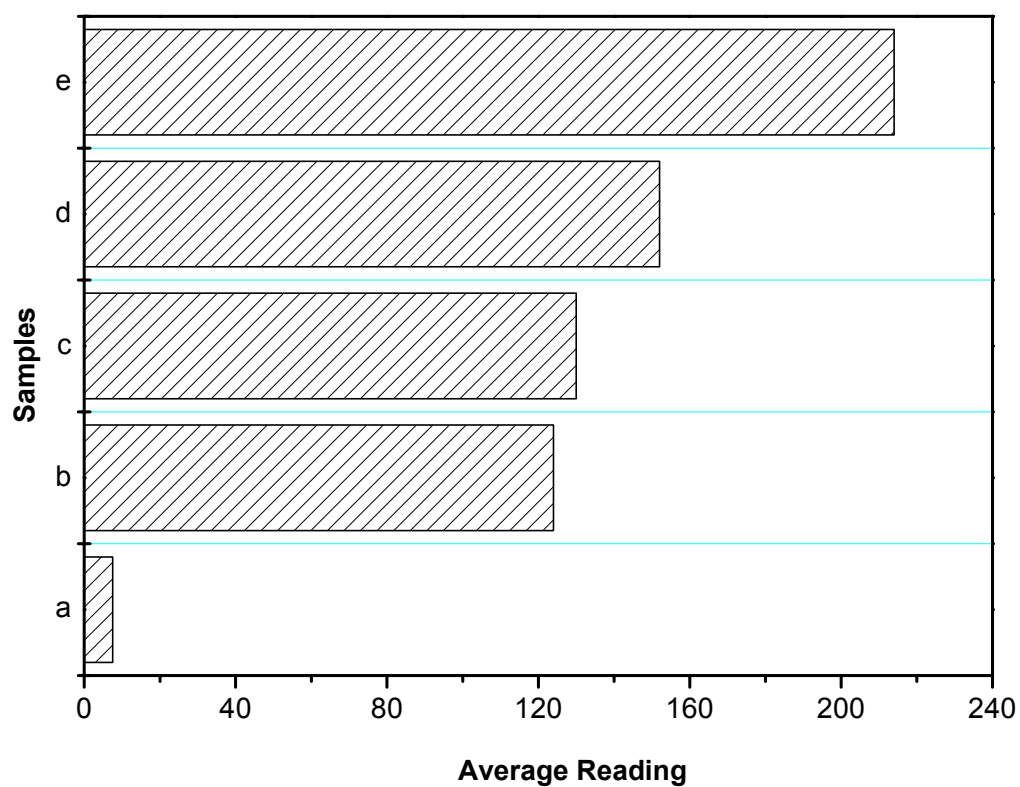


Figure 10. Activity of immobilized OPAA synthesized via the first approach (a) OPAA-F60 and via the second approach (b) TMOS-F50-OPAA, (c) TMOS-PEG50-OPAA, (d) MTMS-PEG50-OPAA, (e) 3MTMS-hydrogel-OPAA. Under the same enzymatic activity assay conditions, the average reading of free OPAA is 381.2.

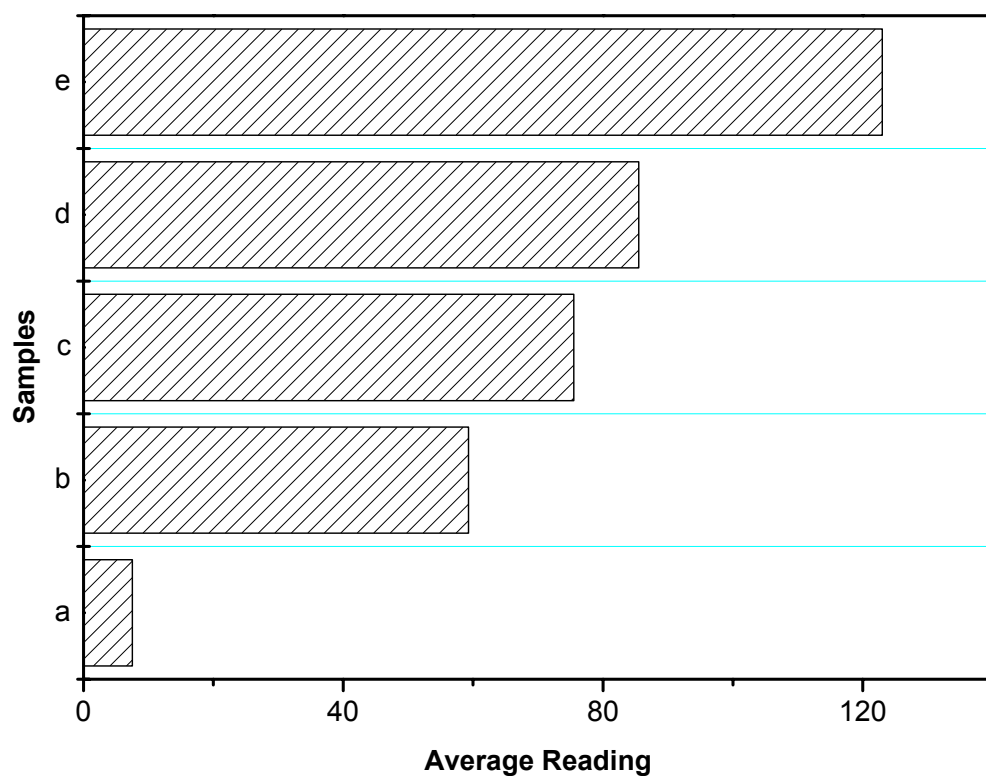


Figure 11. Activity of immobilized OPAA synthesized via the first approach (a) OPAA-F60, and samples synthesized via the second approach after soaking in methanol. (b) TMOS-F50-OPAA, (c) TMOS-PEG50-OPAA, (d) MTMS-PEG50-OPAA, (e) 3MTMS-hydrogel-OPAA.

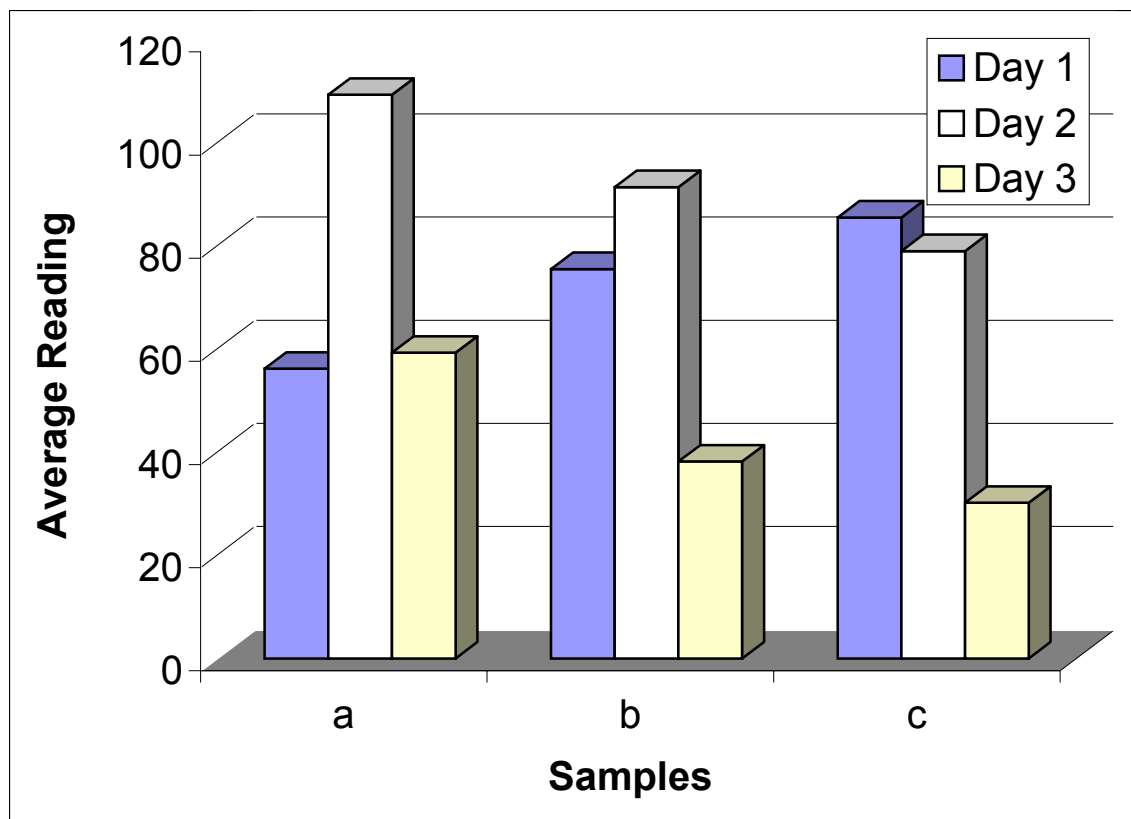


Figure 12. Stability of immobilized OPAA in methanol. (a) TMOS-F50-OPAA (b) TMOS-PEG50-OPAA (c) MTMS-PEG50-OPAA.

6.0. References Cited:

1. Brinker, C. J.; Scherer, G. W. *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*; Academic Press: New York, **1990**.
2. Braun, S.; Rappoport, S.; Zusman, R.; Avnia, D.; Ottolenghi, M. *Mater. Lett.* **1990**, *10*, 1.
3. Avnir, D.; Braun, S.; Lev, O.; Ottolenghi, M. *Chem. Mater.* **1994**, *6*, 1605.
4. Ellerby, L. M.; Bishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Science* **1992**, *255*, 1113.
5. Gill, I.; Ballesteros, A. *J. Am. Chem. Soc.* **1998**, *120*, 8587.
6. Gill, I.; Ballesteros, A. *Trends in Biotech.* **2000**, *18*, 282.
7. Yamanaka, S. A.; Nishida, F.; Ellerby, L. M.; Nishida, C. R.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Chem. Mater.* **1992**, *4*, 495.
8. Dave, B. C.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Anal. Chem.* **1994**, *66*, 1120A.
9. Lin, J.; Brown, C. W. *Trends Anal. Chem.* **1997**, *16*, 200.
10. Tess, M. E.; Cox, J. A. *J. Pharm. Biomed. Anal.* **1999**, *19*, 55.
11. Dunn, B.; Miller, J. M.; Dave, B. C.; Valentine, J. S.; Zink, J. I. *Acta Mater.* **1998**, *46*, 737.
12. Miller, J. M.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Non-Cryst. Solids*, **1996**, *202*, 279.
13. Livage, J.; Roux, C.; Da Costa, J. M.; Desportes, I.; Quinson, J. F. *J. Sol-Gel Sci. Technol.* **1996**, *7*, 45.
14. Reetz, M. T.; Zonta, A.; Simpelkamp, J. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 301.
15. Wang, R.; Narang, U.; Prasad, P. N.; Bright, F. V. *Anal. Chem.* **1993**, *65*, 2671.
16. Pope, E. J. A.; Braun, K.; van Hitum, M.; Peterson, C. M.; Presco, P.; Andrade, J. D. *Living Ceramics. Ceram. Trans.* **1995**, *55*, 33.
17. Narang, U.; Prasad, P. N.; Bright, F. V. *Anal. Chem.* **1994**, *66*, 3139.
18. Lowry, J. P.; McAteer, K.; El Atrash, S. S.; Duff, A.; O'Deill, R. D. *Anal. Chem.* **1994**, *66*, 1754.
19. Guiomar, J.; Guthrie, J. T.; Evans, S. D. *Langmuir*, **1999**, *15*, 1198.

20. Tatsu, Y.; Yamashita, K.; Yamaguchi, M.; Yamamura, S.; Yamamoto, H.; Yoshikawa, S. *Chem. Lett.* **1992**, 1615.
21. Braun, S.; Shtelzer, S.; Rappoport, S.; Avnir, D.; Ottolenghi, M. *J. Non-Cryst. Solids*, **1992**, 147/148, 739.
22. Shtelzer, S.; Braun, S. *Biotechnol. Appl. Biochem.* **1994**, 19, 293.
23. Lev, O.; Glezer, V. *J. Am. Chem. Soc.* **1993**, 115, 2533.
24. Wang, B. Q.; Li, B.; Deng, Q.; Dong, S. *J. Anal. Chem.* **1998**, 70, 3170.
25. Pandey, P. C.; Upadhyay, S.; Pathak, H. C.; Tiwari, I.; Tripathi, V. S. *Electroanalysis* **1999**, 11(17), 1251.
26. Coche-Guérente, L.; Cosnier, S.; Labbé, P. *Chem. Mater.* **1997**, 9, 1348.
27. Yang, S.; Lu, Y.; Atanossov, P.; Wilkins, E.; Long, X. *Talanta* **1998**, 47, 735.
28. Künzelmann, U.; Böttcher, H. *Sensors and Actuators, B*, **1997**, 38-39, 222.
29. Zheng, L.; Reid, W. R.; Brennan, J. D. *Anal. Chem.* **1997**, 69, 3940.
30. Doody, M. A.; Baker, G. A.; Pandey, S.; Bright, F. V. *Chem. Mater.* **2000**, 12, 1142.
31. Wambolt, C. L.; Saavedra, S. S. *J. Sol-Gel. Sci. Technol.* **1996**, 7, 53.
32. Shen, C.; Kosti, N. M. *J. Am. Chem. Soc.* **1997**, 119, 1304.
33. Narang, U.; Prasad, P. N.; Bright, F. V.; Kumar, K.; Kumar, N. D.; Malhotra, B. D.; kamalasanan, M. N.; Chandra, S. *Chem. Mater.* **1994**, 6, 1596.
34. Jordan, J. D.; Dunbar, R. A.; Bright, F. V. *Anal. Chim. Acta* **1996**, 332, 83.
35. Badji, J. D.; Kosti, N. M. *Chem. Mater.* **1999**, 11, 3671.
36. Zheng, L.; Flora, K.; Brennan, J. D. *Chem. Mater.* **1998**, 10, 3974.
37. Flora, K.; Brennan, J. D. *Anal. Chem.* **1998**, 70, 4505.
38. Miller, J. M.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Non-Cryst. Solids* **1996**, 220, 279.
39. (a)Chen, Q.; Kenausis, G. L.; Heller, A. *J. Am. Chem. Soc.* **1998**, 120, 4582. (b) Heller, J.; Heller, A. *J. Am. Chem. Soc.* **1998**, 120, 4586.
40. Brennan, J. D. *Appl. Spectrosc.* **1999**, 53, 106A.
41. Keeling-Tucker, T.; Rakic, M.; Spong, C.; Brennan, J. D. *Chem. Mater.* **2000**, 12, 3695.

42. Dave, B. C.; Dunn, B.; Valentine, J. S.; Zink, J. I. *Nanotechnology: Molecularly designed Materials*, ed. Chow, G. W. and Gonsalves, K. E. ACS, Washington DC, **1995**, p. 351.
43. Arroyo, M.; Moreno, J. M.; Sinisterra, J. V. *J. Mol. Catal.* **1993**, 83, 261.
44. Kuncova, G.; Guglielmi, M.; Dubina, P.; Safar, B. *Collect. Czech. Chem. Commun.* **1995**, 60, 1573.
45. Reetz, M. T.; Zonta, A.; Simpelkamp, J.; Rufinska, A.; Tesche, B. *J. Sol-Gel. Sic. Technol.* **1996**, 7, 35.
46. Reetz, M. T.; Zonta, A.; Simpelkamp, J. *Biotechnol. Bioeng.* **1996**, 49, 527.
47. Kauffmann, C.; Mandelbaum, R. T. *J. Biotechnol.* **1998**, 62, 169.
48. Chen, Q.; Kenausis, G. L.; Heller, A. *J. Am. Chem. Soc.* **1998**, 120, 4582.
49. Heller, J.; Heller, A. *J. Am. Chem. Soc.* **1998**, 120, 4586.
50. Baker, G. A.; Jordan, J. A.; Bright, F. V. *J. Sol-Gel Sci. Technol.* **1998**, 11, 43.
51. Baker, G. A.; Pandey, S.; Maziarz, E. P. III; Bright, F. V. *J. Sol-Gel Sci. Technol.* **1999**, 15, 37.
52. Lesot, P.; Chapuis, S.; Bayle, J. P.; Tault, J.; Lafontaine, E.; Campero, A.; Judeinstein, P. *J. Mater. Chem.* **1998**, 8, 147.
53. Wang, N.; Li, B.; Deng, Q.; Dong, S. *Anal. Chem.* **1998**, 70, 3170.
54. Altstein, M.; Segev, G.; Aharonson, N.; Ben-Aziz, O.; Turniansky, A.; Avnir, D. *Agric. Food Chem.* **1998**, 46, 3318.
55. Flora, K. K.; Dabrowski, M. A.; Musson, S. P.; Brennan, J. D. *Can. J. Chem.* **1999**, 77, 1617.
56. Narang, U.; Jordan, J. D.; Bright, F. V.; Prasad, P. N. *J. Phys. Chem.* **1994**, 98, 8108.
57. Bhatia, R. B.; Brinker, C. J.; Ashley, C. S.; Harris, T. M. *Mater. Res. Soc. Symp. Proc.* 1998, 519, 183.
58. Wei, Y.; Jin, D.; Ding, T.; Shih, W. H.; Liu, X.; Cheng, Z. D.; Fu, Q. *Adv. Mater.* **1998**, 3, 313.
59. Wei, Y.; Xu, J.; Dong, H.; Dong, J. H.; Qiu, K. Y.; Jansen-Varnum, S. A. *Chem. Mater.* **1999**, 11, 2023.
60. Wei, Y.; Xu, J.; Feng, Q.; Dong, H.; Lin, M. *Mater. Lett.* **2000**, 44, 6.
61. Wei, Y.; Xu, J.; Feng, Q.; Lin, M.; Dong, H.; Zhang, W.; Wang, C. *J. Nanosci. Nanotechnol.* **2001**, 1(1), 83-93.

62. Wei, Y.; Xu, J.; Jin, D.; Lin, M.; Feng, Q. *Proc. NAM'99 Tech. Prog. (North Am. Cataly. Soc.)* **1999**, Boston, MA, 14.
63. Xu, J. Ph.D dissertation, Drexel University, **2000**.
64. Xu, J.; Dong, H.; Feng, Q.; Wei, Y. *Polym. Prep.* **2000**, *41*, 1042 (b) Xu, J.; Feng, Q.; Dong, H.; Wei, Y. *Polym. Prep.* **2000**, *41*, 1046. (c) Xu, J.; Dong, H.; Feng, Q.; Wei, Y. *Polym. Prep.* **2000**, *41*(2), 1637.
65. Dumas, D. P.; Caldwell, S. R.; Wild, J. R.; Radshel, F. R. *J. Biol. Chem.* **1989**, *264*, 19659.
66. Defrank, J. J.; Cheng, T. C.; *J. Bacteriol.* **1991**, *173*, 1938.
67. Defrank, J. J.; Beaudry, W. T.; Cheng, T. C.; Flarvey, S. P. Stroup, A. N. Szaafraniec, L. L. *Chem. Biol. Interact.* **1993**, *87*, 141.
68. Cheng, T. C.; Rastogi, V. K.; Defrank, J. J. Sawiris, G. P. *Enzyme Engineering XIV*, **1998**, *864*, 253.
69. Cheng, T. C.; Harvey, S. P.; Chen, G. L. *Appl. Environ. Microbiol.* **1996**, *62*(5), 1636.
70. Cheng, T. C.; DeFrank, J. J.; Rastogi, V. K. *Chemico-Biolog. Interactions.* **1999**, *119*, 455.
71. Barrett, E. P.; Joyner, L.; Halenda, P. P. *J. Am. Chem. Soc.* **1951**, *73*, 373.
72. Jin, D. L. Ph.D dissertation, Drexel University, **1997**.
73. C&EN, P52, August 30, **1999**
74. van Mierlo, C. P. M.; Steensma, E. *J. Biotech.* **2000**, *79*, 281.
75. Rozema, D.; Gellman, S. H.; *J. Am. Chem. Soc.* **1995**, *117*, 23733.